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VOLUME IV NUMBER 1

# STAIN TECHNOLOGY

JANUARY, 1929

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# STAIN TECHNOLOGY

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*Editor and Business Manager*
H. J. CONN
Soil Bacteriologist, N. Y. Agr. Exp. Station

*Consulting Editors*
CHEMISTRY: W. C. HOLMES Color Lab., U. S. Dept. of Agriculture
ZOOLOGY: S. I. KORNHAUSER School of Medicine, Univ. of Louisville
PATHOLOGY: F. B. MALLORY Boston City Hospital
BOTANY: W. R. TAYLOR Univ. of Pennsylvania

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Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

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# STAIN TECHNOLOGY

VOLUME IV JANUARY, 1929 NUMBER 1

## PROGRESS IN THE STANDARDIZATION OF STAINS

### STAIN SPECIFICATIONS

It was pointed out in this journal a few years ago (French 1926)[1] that government departments have a practical problem involved in assigning specifications for stains which is quite different from that which the Stain Commission has been trying to meet. Two conferences have been held within the last six months which seem likely to harmonize the two points of view and secure close coöperation in the matter in the future.

The specifications drawn up by the Stain Commission have merely been intended to secure a product that would probably work satisfactorily, and upon which the Commission certification label could be placed if the actual tests of the sample were favorable. From the standpoint of the purchaser the main emphasis has been upon the certification label of the Commission, not on the published specifications.

In the case of the government purchasing agencies, as pointed out by French, the procedure has to be entirely different. The government, as a purchaser, has to place its reliance upon specifications and can not specify a stain certified by the Commission. As a matter of fact any company trying to sell a stain to the government can claim that their product, altho not certified, is just as good as the certified stain, and the government may have great difficulty in proving the contrary. For this reason it has been evident that the government should draw up specifications for stains which would assure a good product without excluding any stain that would be satisfactory in actual use. The Commission's specifications, as pointed out by French, have never been adequate for this purpose.

To secure specifications of the type necessary for the government has meant considerable work. The Stain Commission would gladly have published such specifications in the first place had the necessary information been available. Since this information was not at hand, the certification method of securing reliable samples was adopted. At present, however, the Army Medical Museum, in coöperation with the Color Laboratory of the Department of Agriculture, has been obtaining the necessary data in connection with several stains. This information is being placed at the disposal of a

[1]French, R. W., Stain Techn. 1, 11–16. 1926.

committee of the Army, who plan to submit the specifications when they are prepared to the Surgeon General's office for adoption by the Medical Department. It is quite possible that these stain specifications will be adopted subsequently by the Federal Specifications Board. These specifications are being drawn up in close coöperation with the Biological Stain Commission. This coöperation is being assured thru the fact that the data for the army specifications are being obtained by R. W. French of the Army Medical Museum, who is a member of the executive committee of the Commission. As a further aid to this coöperation two conferences have been held at which the Chairman of the Commission was present.

When these specifications are adopted the matter will be handled so as to make use of as much as possible of the machinery already built up by the Stain Commission for testing and approving stains. At present when any stain sample is submitted to the Commission, part of it is always sent to the Color Laboratory of the Department of Agriculture for optical and chemical tests. In the future the Color Laboratory will determine whether any such sample meets the government specifications in instances where such specifications have been drawn up. If it does not meet these specifications, it will not necessarily be refused certification, provided it proves satisfactory in actual use, but in either event the information as to whether or not it meets the specifications will be furnished to the proper government agencies. If subsequently the stain company offers a lot of dye bearing this certification number to any department of the government for which these specifications apply, it will be known automatically whether the dye meets them. In this way the government will be able to give a practical recognition to the work of this Commission in standardizing stains, even tho they are not able to specify Commission certified stains outright. The value of such recognition is obvious, and it is a matter of much gratification that arrangements of this sort are likely to be perfected.

H. J. Conn

## Stains Recently Certified

It has been pointed out that certain information in the possession of the Commission as to the batches of stains that have been certified may be of value to the users of dyes. It is accordingly planned to publish each quarter a list of the stains approved for certification and their dye content, wherever a determination of the latter is possible. The list below covers a period of six months—June to November 1928—; but in the future such lists will each cover three months only.

STAINS CERTIFIED JUNE TO NOVEMBER, 1928*

| Name of dye | Certification No. of batch | Dye Content | Objects of tests made by Commission† | Date approved |
|---|---|---|---|---|
| Wright's stain | NWr 5 | —— | As blood stain | June, 1928 |
| Congo red | NQ 2 | 89% | As histological counter-stain | June, 1928 |
| Neutral red | CX 2 | —— | For vital staining | June, 1928 |
| Light green SF yellowish | CL 2 | 67% | As cytological counter-stain | June, 1928 |
| Janus green | CJ 2 | 46% | As vital stain for blood | June, 1928 |
| Eosin Y | NE 3 | 90% | As histological counter-stain | July, 1928 |
| Eosin Y | LE 4 | 75% | As histological counter-stain | Sept., 1928 |
| Fuchsin, basic | CF 8 | 92% | For staining tubercle bacillus, and for use in Endo medium | Oct., 1928 |
| Nigrosin | NNi 5 | —— | As background stain, especially in Dorner spore stain | Oct., 1928 |
| Pyronin | NP 3 | —— | As constituent of Pappenheim stain | Nov., 1928 |
| Eosin B | NEb 3 | 90% | As histological counter-stain | Nov., 1928 |

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples here listed may be employed. The Commission ordinarily tests each dye for such of its ordinary uses as seem to give the most severe check as to its practical value.

## PRESENT MEMBERS OF THE COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS

The latest published list of the Commission members appeared in January, 1926. There has been considerable change in the membership and in the addresses of the old members who are still on the commission. The following is an up-to-date list:

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J. W. Churchman, 414 E. 26th St., New York City.
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R. B. H. Gradwohl, 3514 Lucas Ave., St. Louis, Mo.
F. W. Hachtel, 122 W. Lafayette Ave., Baltimore, Md.
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M. J. Harkins, 2101 Pine St., Philadelphia, Pa.
F. C. Harrison, Path. Inst., McGill University, Montreal, Canada.
D. J. Healy, Experiment Station, Lexington, Ky.
H. T. Herrick, Bureau of Chem. & Soils, Washington, D. C.
W. A. Hinton, 240 Longwood Ave., Boston, Mass.
M. J. Hogue, Med. Lab., Univ. of Pennsylvania, Philadelphia, Pa.
W. C. Holmes, Color Laboratory, Dept. of Agriculture, Washington, D. C.
E. G. Hood, Dairy & Cold Storage Branch, Ottawa, Canada.
Davenport Hooker, Univ. of Pittsburgh, Pittsburgh, Pa.
Charles A. Hunter, State Health Laboratory, Vermillion, S. D.
Gemma Jackson, Botany Dept., Cornell University, Ithaca, N. Y.

Leon S. Lippincott, Box 303, Vicksburg, Miss.
H. Macy, University Farm, St. Paul, Minn.
M. S. Marshall, Hooper Foundation, 2nd & Parnassus Aves., San Francisco, Cal.
Barbara McClintock, Botany Dept., Cornell University, Ithaca, N. Y.
C. E. McClung, Univ. of Pennsylvania, Philadelphia, Pa.
J. W. Miller, Charity Hospital, New Orleans, La.
John T. Myers, Univ. of Nebraska, College of Medicine, Omaha, Neb.
E. M. Pickens, College Park, Md.
R. O. Pierson, Health Dept., 441 Main St., E. Orange, N. J.
L. F. Randolph, Dept. Botany, College of Agriculture, Ithaca, N. Y.
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Alexander V. Tolstoouhov, Sydenham Hospital, 565 Manhattan Ave., New York City.
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B. G. R. Williams, Paris, Ill.

# THE ATMOSPHERIC OXIDATION, OR DEALKYLATION, OF AQUEOUS SOLUTIONS OF METHYLENE BLUE

W. C. Holmes and E. F. Snyder, (*Contribution No. 152 from Color and Farm Waste Division, jointly with Division of Soil Fertility Investigations, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.*)

Abstract.—**At room temperatures (approximately 25° C) dilute aqueous solutions of representative samples of methylene blue are stable below pH 9.5. Oxidation, or dealkylation, begins between pH 9.5 and 9.8 and increases in both rate and degree with increasing alkalinity. Below pH 11 the rate is slow and the total oxidation relatively limited. At pH 12, however, oxidation is rapid and is practically complete within a few days.**

**Trimethyl thionin is decidedly less stable to atmospheric oxidation than is methylene blue, and undergoes appreciable dealkylation at moderate alkalinities which do not affect the latter dye.**

It has been known for many years that methylene blue undergoes atmospheric oxidation, or dealkylation, in alkaline aqueous solutions.[1] The initial reaction product formed is trimethyl thionin (together with formaldehyde) while asymmetrical dimethyl thionin and dimethyl thionolin (the methylene violet of Bernthsen) are obtained subsequently. The precise conditions necessary for the atmospheric oxidation of methylene blue, however, have never been determined and the need for definite information on that point has become increasingly urgent as the scientific applications of the dye have multiplied. It is now recognized that the essential properties of methylene blue in numerous applications are decidedly different from those of its oxidation products and that further data are desirable on factors which influence the stability of its solutions.

Preliminary tests were made by preparing dilute solutions of methylene blue in buffered solutions of sea water, ageing them with access to air, and withdrawing aliquots from time to time for examination. The analytical method employed was that of determining the ratio of extinction coefficients at 640 and 670 mm., after dilution with an equal volume of an organic solvent.[2] With strongly alkaline solutions of the dye, acetic acid was found to be a much more satisfactory diluent than alcohol. The method was originally

[1] F. Kerhmann, E. Havas und E. Grandmougin. Über Farbbasen der Chinonimid-Farbstoffe. Ber. d. d. Chem. Ges., **46**, 2131 1913.

[2] W. C. Holmes. The Spectrophotometric Evaluation of Mixtures of Methylene Blue and Trimethyl Thionin, Stain Techn., **3**, 45 1928.

designed for the evaluation of mixtures of methylene blue and trimethyl thionin. In the present application it proved an excellent means for detecting incipient oxidation of methylene blue and for following the course of the oxidation. It should be noted, however, that absorption ratios obtained in oxidation tests on methylene blue do not afford an entirely reliable index of the relative proportions of that dye and of trimethyl thionin, owing to the progressive formation of lower homologs in the same solutions.

In the preliminary tests it was found that methylene blue was decidedly unstable in hundredth normal sodium hydroxide, undergoing practically complete oxidation within a few days. At pH 9.8 evidence was obtained of its oxidation in a very minor degree after long standing. Under the same conditions trimethyl thionin was oxidized somewhat rapidly. The latter dye also underwent considerable oxidation at pH 9.0 in the course of a month, and was appreciably affected even at pH 7.8. Solutions of methylene blue, however, at pH 8.7 and pH 6.9 showed no appreciable oxidation even after a period of two months.

A second series of tests was then conducted with methylene blue. Two dyes were employed, one of which (A) was a certified biological stain and the other (B) was synthesized at the Color Laboratory from pure intermediates. A series of solutions of known hydrogen-ion concentrations was prepared by diluting a stock solution of dye of suitable concentration in distilled water with an equal volume of twentieth molar boric acid, potassium chloride, sodium hydroxide buffer solutions. The resulting solutions were examined immediately and re-examined at intervals for a month. In their examination 5-cc. aliquots were diluted with an equal volume of acetic acid and the extinction coefficients measured in a 2-cm. layer. A Hilger wavelength spectrometer provided with a Nutting photometer was employed. The data from these tests are recorded in Table 1.

The general decrease in absorption values (E) which occurs in such solutions on standing is taken as evidence of physical rather than chemical alteration. It is attributed to increasing molecular aggregation of the dye brought about by the inorganic salts present. Alteration in absorption ratios (R), on the other hand, is indicative of oxidation, or dealkylation. The absorption ratio of both dye samples under the experimental conditions is approximately 0.75. The probable experimental error is less than 0.03, so that it appears safe to conclude that an absorption ratio of 0.78 or higher constitutes positive evidence of oxidation. This ratio corresponds to a conversion of approximately 3% of the methylene blue to trimethyl thionin.

**Table 1. Absorption Ratios of Aged Methylene Blue Solutions.**

| Time | Dye Sample | pH 7.97 | | | pH 8.98 | | | pH 9.49 | | | pH 10.02 | | | pH 10.37 | | | pH 10.87 | | | pH 11.43 | | | pH 12 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | E at 640 | E at 670 | R | E at 640 | E at 670 | R | E at 640 | E at 670 | R | E at 640 | E at 670 | R | E at 640 | E at 670 | R | E at 640 | E at 670 | R | E at 640 | E at 670 | R | E at 640 | E at 670 | R |
| Immediate | A | 1.52 | 2.03 | .75 | 1.52 | 2.03 | .75 | 1.54 | 2.08 | .74 | 1.53 | 2.07 | .74 | 1.51 | 2.01 | .75 | 1.52 | 2.00 | .76 | 1.50 | 2.01 | .75 | 1.24 | 1.63 | .76 |
| | B | 1.47 | 1.94 | .76 | 1.43 | 1.91 | .75 | 1.47 | 1.95 | .75 | 1.42 | 1.90 | .75 | 1.49 | 2.00 | .745 | 1.44 | 1.94 | .74 | 1.47 | 1.96 | .75 | 1.16 | 1.55 | .75 |
| 1 Day | A | 1.48 | 2.00 | .74 | 1.46 | 1.97 | .74 | 1.48 | 2.03 | .73 | 1.46 | 1.97 | .74 | 1.44 | 1.95 | .74 | 1.50 | 2.00 | .75 | 1.38 | 1.84 | .75 | 0.76 | 0.75 | 1.01 |
| | B | 1.42 | 1.92 | .74 | 1.38 | 1.87 | .74 | 1.40 | 1.89 | .74 | 1.38 | 1.88 | .745 | 1.41 | 1.90 | .74 | 1.41 | 1.91 | .74 | 1.32 | 1.725 | .765 | 0.72 | 0.75 | .96 |
| 2 Days | A | 1.45 | 1.95 | .75 | 1.45 | 1.96 | .74 | 1.45 | 1.96 | .74 | 1.42 | 1.92 | .74 | 1.38 | 1.86 | .74 | 1.41 | 1.85 | .76 | 1.20 | 1.52 | .79 | 0.57 | 0.475 | 1.20 |
| | B | 1.39 | 1.87 | .74 | 1.37 | 1.85 | .74 | 1.38 | 1.87 | .74 | 1.35 | 1.82 | .74 | 1.35 | 1.82 | .74 | 1.34 | 1.81 | .74 | 1.15 | 1.43 | .80 | 0.54 | 0.46 | 1.17 |
| 3 Days | A | 1.43 | 1.92 | .75 | 1.42 | 1.92 | .74 | 1.42 | 1.92 | .74 | 1.40 | 1.87 | .75 | 1.32 | 1.76 | .75 | 1.35 | 1.75 | .77 | 1.05 | 1.24 | .85 | 0.40 | 0.25 | 1.60 |
| | B | 1.39 | 1.86 | .75 | 1.35 | 1.80 | .75 | 1.36 | 1.81 | .75 | 1.33 | 1.79 | .74 | 1.28 | 1.72 | .745 | 1.27 | 1.68 | .76 | 1.01 | 1.17 | .86 | 0.38 | 0.24 | 1.58 |
| 5 Days | A | 1.40 | 1.90 | .74 | 1.40 | 1.87 | .75 | 1.40 | 1.90 | .74 | 1.37 | 1.85 | .74 | 1.28 | 1.69 | .76 | 1.30 | 1.63 | .80 | 0.89 | 0.96 | .93 | —— | —— | —— |
| | B | 1.38 | 1.86 | .74 | 1.33 | 1.77 | .75 | 1.33 | 1.80 | .74 | 1.31 | 1.75 | .75 | 1.25 | 1.67 | .75 | 1.24 | 1.55 | .80 | 0.84 | 0.88 | .95 | 0.355 | 0.22 | 1.60 |
| 1 Week | A | 1.39 | 1.85 | .75 | 1.36 | 1.80 | .76 | 1.34 | 1.81 | .74 | 1.33 | 1.71 | .78 | 1.27 | 1.61 | .79 | 1.22 | 1.47 | .83 | 0.83 | 0.77 | 1.08 | —— | —— | —— |
| | B | 1.38 | 1.84 | .75 | 1.30 | 1.74 | .75 | 1.28 | 1.71 | .75 | 1.27 | 1.65 | .77 | 1.23 | 1.57 | .78 | 1.15 | 1.40 | .82 | 0.79 | 0.72 | 1.09 | —— | —— | —— |
| 2 Weeks | A | 1.39 | 1.86 | .74 | 1.29 | 1.70 | .76 | 1.26 | 1.68 | .75 | 1.18 | 1.50 | .79 | 1.12 | 1.38 | .81 | 1.05 | 1.21 | .87 | 0.55 | 0.40 | 1.36 | —— | —— | —— |
| | B | 1.37 | 1.85 | .75 | 1.24 | 1.64 | .76 | 1.20 | 1.60 | .75 | 1.13 | 1.43 | .79 | 1.03 | 1.30 | .80 | 0.99 | 1.15 | .86 | 0.51 | 0.375 | 1.36 | —— | —— | —— |
| 3 Weeks | A | 1.34 | 1.76 | .76 | 1.26 | 1.68 | .75 | 1.09 | 1.47 | .74 | 1.08 | 1.21 | .81 | 1.04 | 1.27 | .82 | 0.90 | 1.03 | .87 | 0.42 | 0.28 | 1.48 | —— | —— | —— |
| | B | 1.35 | 1.80 | .75 | 1.21 | 1.58 | .76 | 1.04 | 1.37 | .76 | 1.03 | 1.29 | .80 | 0.98 | 1.20 | .82 | 0.84 | 0.97 | .87 | 0.38 | 0.26 | 1.46 | —— | —— | —— |
| 4 Weeks | A | 1.30 | 1.71 | .76 | 1.25 | 1.64 | .76 | 1.02 | 1.38 | .74 | 1.01 | 1.25 | .81 | 0.95 | 1.16 | .82 | 0.75 | 0.85 | .88 | 0.36 | 0.24 | 1.49 | —— | —— | —— |
| | B | 1.27 | 1.71 | .74 | 1.20 | 1.57 | .76 | 0.98 | 1.30 | .75 | 0.96 | 1.19 | .81 | 0.90 | 1.09 | .83 | 0.67 | 0.77 | .87 | 0.335 | 0.22 | 1.48 | —— | —— | —— |

Solutions of the dye appear stable at and below pH 9.5. Definite evidence of oxidation is obtained at pH 10.02 in from one to two weeks, at pH 10.37 in one week, at pH 10.87 in five days, at pH 11.43 in two days and at pH 12 within a few hours. Apparently an equilibrium is reached within a few weeks, and the total degree of oxidation of the dye, as well as its rate of oxidation, is dependent upon the hydrogen-ion concentration of the solution, the oxidation increasing with increasing alkalinity. Below pH 11 the rate of oxidation, or dealkylation, is slow and only a relatively minor proportion of the dye undergoes oxidation.

The conditions of the investigation proved unfavorable for the study of the precise mechanism of the reactions involved, but it is hoped that such a study may be undertaken later. The great ease with which aminated dyes of the type of methylene blue undergo atmospheric oxidation, or dealkylation, in alkaline aqueous solutions appears remarkable. The senior writer has shown that such dyes exist in aqueous solutions in two tautomeric forms, for one of which he has predicated a structure in which one alkyl group is bound to the amino nitrogen atom merely by partial valencies.[3] This hypothesis affords a plausible explanation of the ease with which dealkylation is effected, irrespective of the precise mechanism of the reaction.

[3]Holmes, W.C. The Chemical Nature of Metachromasy. Stain Techn., 1, 116. 1926.

# ELASTIC TISSUE STAINING[1]

R. W. French, *Army Medical Museum, Washington, D. C.*

Abstract.—**It has been found that the addition of dextrin to samples of crystal violet and basic fuchsin employed in the preparation of the elastic tissue stain after the technic of Weigert makes more sure a satisfactory final product. A modification of the original Weigert technic employing crystal violet or a mixture of crystal violet and basic fuchsin is offered as providing a better color contrast both visually as well as photographically. Crystal violet alone affords a bright greenish-yellow elastin while the addition of basic fuchsin results in a darker stain shading into dark blue as the proportion of basic fuchsin is increased.**

Difficulty was early encountered in the preparation of an elastic tissue stain after the technic proposed by Prince[2] as a modification of Weigert's[3] resorcin-fuchsin elastic stain. When satisfactory results were obtainable with the Prince modification, employing crystal violet in the place of basic fuchsin, the resultant picture was splendid with a bright yellowish green elastin. However, it was found that only certain earlier samples of crystal violet would give consistent results in producing a satisfactory dye and it was thought possible that the addition of dextrin might aid in the reaction. This was tried and found to aid in obtaining consistent results irrespective of the source of the crystal violet. The following technic of employment and preparation of the stain was finally determined upon:

1. Bring sections affixed to the slide into water.
2. Stain in the modified Weigert elastic stain prepared as follows for ½ to 3 hours:

| | |
|---|---|
| Crystal violet | 2.0 gms. |
| Dextrin | 0.5 gm. |
| Resorcinol | 4.0 gms. |
| Water, distilled | 200.0 ml. |

Boil in a porcelain dish. When boiling briskly add 25 ml. of 29% aqueous ferric chloride, stir and continue boiling for 2 to 5 minutes. A heavy precipitate forms and the mixture assumes a greenish cast. Cool and filter. Boil the precipitate in 200 ml. of 95% ethyl alcohol

[1]Read before the 21st annual meeting of the American and Canadian Section of the International Association of Medical Museums at Washington, D. C., April 30, 1928.

[2]Prince, L. H. Elastic Fiber Stain. U. S. Veterans' Bureau, Med. Bul. 3, (No. 1) 83. Washington, 1927.

[3]Weigert. Centralb. allg. Path., 9, 290. 1898.

over a water bath or electric hot plate. Cool, filter and make up to 200 ml. Add 4 ml. of concentrated hydrochloric acid.

3. Differentiate in 95% alcohol.
4. Wash in water.

As a routine procedure, preparations are then stained with the Weigert iron hematoxylin or Harris alum hematoxylin and counterstained with the Van Gieson mixture of picric acid and acid fuchsin mixture. Almost any desired stain may be used however, after the elastic stain. The staining is selective, only elastin being stained a brilliant yellowish green.

No particular reason can be ascribed to the employment of the dextrin. It has been found necessary for consistent results, however, especially with a sample of crystal violet which had been purified by recrystallization. It is possible that it aids in the reaction between the dye, resorcin, and ferric chloride, as a catalytic agent, or in some similar manner. The chemistry of the production of the elastin dye is obscure and little is known as to the probable product or particular dye resulting in the selective staining of the elastin from a chemical standpoint.

After employing this procedure in the staining of elastic tissue for some time, it became necessary to prepare photomicrographs showing elastic changes and this stain with the Weigert iron hematoxylin and Van Gieson counterstains was lacking in satisfactory photographic contrasts. In view of the fact that elastin stained with the original basic fuchsin technic of Weigert is black and not easily differentiated photographically from the nuclear stain of hematoxylin it appeared logical to believe that a mixture of basic fuchsin and crystal violet might afford a more desirable stain with suitable contrast for both visual and photographic work. Such was found to be true. Finally a mixture of equal parts of crystal violet and basic fuchsin (rosanilin hydrochloride) has been employed. This results in fairly dark bluish green elastin and has visual as well as photographic contrast when properly filtered. Best photographic results have been obtained with panchromatic plates with an orange (A) filter.

# DOUBLE STAINING OF MITOCHONDRIA AND BACTERIA IN PLANT TISSUES

J. DUFRENOY, *Station Centrale de Phytopathologie et de Parasitologie, Paris, France*

Milovidov, in Professor Guilliermond's laboratory, has worked out the following technic to stain mitochondria red and bacteria blue in root nodules of legumes.

1. Kill tissues for 24 hours in a mixture of 1% chromic acid (50 cc.), 1% potassium dichromate 50 cc., 40% formalin (neutralized by adding powdered calcium carbonate) 8 cc.
2. Rinse 24 hours in running water, dehydrate, imbed, and cut very thin sections (4$\mu$).
3. Dissolve paraffin from slides, dip slides into very thin solution of collodion in absolute alcohol and ether. Run thru series of alcohol and water.
4. Stain slides, at temperature, of about 80°C. in solution:
   Acid fuchsin 2 g.
   Anilin water 10 g.
5. Wash in running water.
6. Destain for a few seconds in:
   Aurantia 0.5 g.
   70% alcohol 100 g.
7. Wash in water.
8. Treat for a few minutes in:
   Phospho-molybdenic acid 1 g.
   Sodium hydroxide, 1% solution 10 g.
   Water 90 g.
9. Rinse in water.
10. Stain for a few minutes in a solution of Unna's polychrome methylene blue diluted in three parts of water.[1]

The writer has been applying that technic to the staining of *Dolichos lablab*, Linn. (the black bean) inoculated with *Phytomonas phaseoli* (Erwin Smith) Bergey et al. (cause of bacterial blight of beans) and made available thru Dr. Burkholder's courtesy. There was obtained a very sharp definition of the bacteria, which stained a deep violet blue, while mitochondria and plastids retained the red acid fuchsin stain.

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[1]Milovidov, P. F. Sur la question de la double coloration des Bactéries et des chondriosomes. C. R. Soc. Biol., **98**, 555–558. 1928.

Fig. 1–2. Longitudinal sections of stem of *Dolichos lablab* L.
B. Bacteria.
m. Mitochondria (staining red).
P. Plastids (staining red, starch, where present, may stain blue).
N. Nuclei.

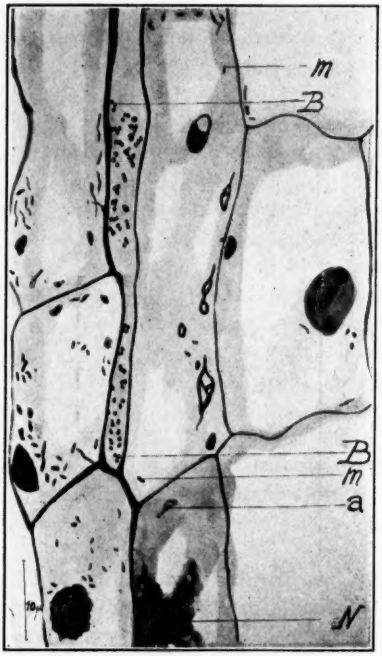


Fig. 1. Bacterial strands pushing between cells. Bacteria show as small isolated colonies.

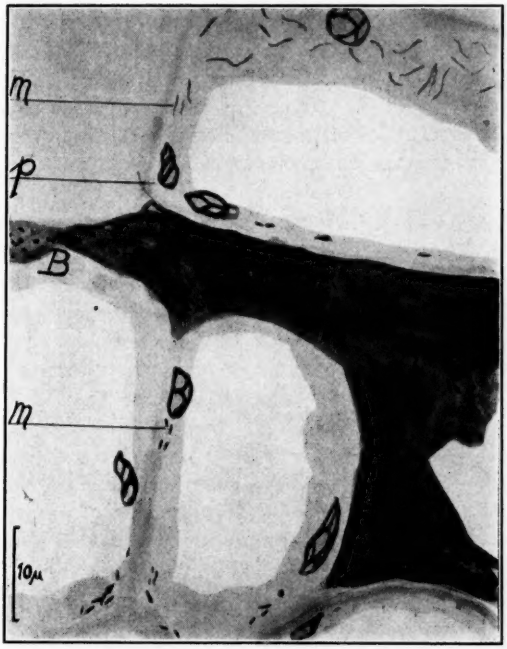


Fig. 2. Older stage of infection; bacteria show as streptococci in zoogleae.

# NATURAL COLOR PRESERVED IN SECTIONED GREEN PLANT TISSUE

E. A. Jirouch, *Biological Laboratory, Western Reserve University, Cleveland, Ohio.*

Abstract.—**Tissues of green plants when mounted after sectioning usually appear very artificial and lifeless. This paper reports a method of retaining the chloroplasts in natural colors and appearance.**

**Tissues were fixed in Keefe's "Preserving fluid for green plants" and then sectioned after freezing them in a 20% gum arabic solution. They were finally mounted in glycerin jelly. Addition of a light stain, as with Delafield's hematoxylin, brings out the details of certain structures, such as the nucleus, without altering the green appearance of the chloroplasts.**

**Such preparations when studied under the microscope give a very neat representation of a green leaf section as it appears in the natural condition.**

The tissue of green plants as fixed, sectioned, and mounted by ordinary methods, lose their natural color, and cannot readily be stained suitably to replace again the lost color. Many fixatives destroy the green pigment, chlorophyll, and produce, in the tissue, a lifeless, dull brown color. Such preparations must, when mounted, be stained and counterstained at times even in gaudy colors, for differentiation of the various structures. The tissues, when thus preserved, appear very artificial. The chlorophyll in the plant cells has disappeared. However, application of a special 'Preserving Fluid for Green Plants' has made possible the retention of a natural appearing chlorophyll derivative in the tissue.

Keefe[1] gave the formula for a plant staining and fixing solution which will preserve the fixed material in the same green condition in which it was before fixation.

The color producing substances in the fixative are uranium nitrate and copper chloride. From the behavior of specimens in this solution, according to Keefe, it may be presumed that there is a reorganization of the chlorophyll with the resulting formation of copper and uranium derivatives in the chloroplasts. Specimens, when fixed in this solution, keep their green color even after prolonged exposure to sunlight. The tissue must be left in the fixative for at least forty-eight (48) hours before it can be used. It can then be stored in this solution and removed as needed, since the fixative has no deteriorating effect on the tissue no matter how long it is left in it.

[1]Keefe, A. M. A preserving fluid for green plants. Science **64**, 331. 1926.

Amaryllis leaves subjected to the fixative were sectioned and mounted according to customary paraffin technic. Inspection of the finished slides showed that the color had left the plant cells almost entirely. A careful study of this difficulty led to the conclusion that the alcohols, used for dehydration, had washed out the chlorophyll substitute. Once the stained tissue had been subjected to alcohol and lost its color, no amount of treatment with the fixative would replace the lost color. Thus the value of the stain depends on the presence of chlorophyll. Since chlorophyll and, presumably, its copper-uranium derivative are soluble in alcohol, any technic requiring alcohol in procedure is worthless here.

The freezing method, not requiring alcohol in procedure, was then tried. The fixed specimens were washed well in repeated changes of distilled water and then placed in a solution of gum arabic, made by dissolving five grams of this substance in twenty-five cubic centimeters of distilled water, till impregnation was complete, a process requiring about two days. (This step is taken so that on freezing the tissue, the cell walls will not explode due to the expansion of the water when forming ice. Gum Arabic expands little if at all when it freezes.) Using a freezing microtome with carbon dioxide attachment, sections about 25–35$\mu$ thick were cut, washed, and mounted in glycerin for examination. Inspection under the microscope showed that the chloroplasts had retained their color perfectly, but that some of them had undergone plasmolysis. This however was remedied by first fixing the leaf in half strength fixing solution, (distilled water as a diluting agent), for about a day and then transferring it to full strength solution until used. Inspection also showed that the epidermal cells of the leaf and the nuclei of the chlorenchyma tissue remained colorless. To bring these out the washed sections were treated with dilute Delafield's hematoxylin (5 c. c. strong stain diluted to 100 c. c. with distilled water) for about two minutes. They were then washed in distilled water containing a little sodium bi-carbonate to turn the hematoxylin to its blue color. The sections were then covered with glycerin to keep them from putrifying before they could be utilized.

For permanent mounting the sections were mounted in glycerin jelly. After all of the air bubbles had been removed, all excess gel was wiped away from the edges of the cover glass, and a protective ring of gold size painted around the edges. (Duco can also be used here.)

Such slides when studied under the microscope, gave a very neat representation, except for the lavender counterpart, of a green leaf

section as it appears in the natural state. The various structures were brought out clearly for study by elementary students.

### Outline of Procedure

1. Tissue placed in half strength fixative for 12 hrs. (Distilled water used as diluting agent.)
2. Transfer to full strength solution for 48 hrs. or till ready for use.
3. Wash well in repeated changes of distilled water to remove all acids and salts, or the knife blade on the microtome will become badly stained with copper during the sectioning process.
4. Place in 20% gum arabic solution for 1 to 2 days.
5. Freeze section to microtome plate using enough gum arabic solution to wall in the section thoroly. Begin cutting just before the frozen mass has started to melt. Practise is the best teacher here. Remove cut sections from blade with a wet camel's hair brush. Sections thicker than 25 to 30$\mu$ are usually more than one cell layer thick. A keen blade frequently sharpened during the cutting will give the best results.
6. Wash sections by decantation with distilled water to remove all gum arabic.
7. Transfer to dilute Delafield's Hematoxylin, 5% solution by vol., for approximately 2 minutes.
8. Wash stained sections in distilled water containing a little sodium bi-carbonate.
   (a) This method of staining the sections "obviates" the usual destaining methods.
9. Place sections into commercial glycerin till they are to be used.
10. Mount in glycerin gel as per usual methods.

# SOME STAINING METHODS FOR BACTERIA AND YEASTS

W. E. Maneval, *Department of Botany, University of Missouri, Columbia, Mo.*

Abstract.—**For staining flagella of bacteria use actively motile organisms 20 to 24 hours old, allow to diffuse in sterile water 20 to 30 minutes, transfer droplets of the suspension to clean slides and let evaporate without spreading. Then treat 2 to 4 minutes with the following mordant: tannic acid 10 or 20%, 50 cc.; ferric chloride 5%, 10 to 15 cc.; carbol fuchsin (Ziehl-Nielson), 5 cc.; hydrogen peroxide 3%, 6 to 8 cc. Wash and stain 2 to 3 minutes with a mixture of basic fuchsin, saturated alcoholic, 10 cc.; anilin oil (1 part) and 95% alcohol (3 parts) mixed, 5 cc.; distilled water, 30 cc.; acetic acid, 4%, 1 cc. Wash thoroly with water.**

**In some kinds of yeast the nucleus may be demonstrated by fixing smears with heat, staining one minute with aqueous acid fuchsin, followed by 5% tannic acid for 20 seconds and finally washing with acidulated water.**

**Attention is called to some of Gutstein's methods of staining bacteria and yeasts. Those methods that the writer has used considerably and found satisfactory for staining vegetative cells and spores of bacteria and yeasts, and for capsules of bacteria, are briefly described. Certain modifications of these methods are mentioned. Boiling of bacterial spores in water for 10 minutes before staining is recommended.**

The staining of bacterial flagella is often omitted in classwork in general bacteriology. However, even for the beginner, such staining is probably as important as the staining of endospores or the Gram reaction. Most methods of staining flagella are rather complicated. It is often difficult to have cultures in an actively motile condition when needed. The mordants and stains used may require considerable time for preparation and sometimes change rapidly afterwards. Other difficulties might be mentioned but they have been discussed recently by Wright (1928). The writer has developed the following method of staining flagella, by which some of these difficulties have been eliminated. It is believed that this method will be found satisfactory for general use with beginners in bacteriology.

### Mordant

| | |
|---|---|
| Tannic acid, 10 or 20% | 50 cc. |
| Ferric chloride, 5% | 10 or 15 cc. |
| Carbol-fuchsin (Ziehl-Nielson) | 5 cc. |
| Hydrogen peroxide, 3% | 6—8 cc. |

Filter the solution of tannic acid. Add the ferric chloride slowly, shaking constantly, and then add the carbol fuchsin. Boil slowly for 2 minutes and filter. Add the hydrogen peroxide, shake frequently for a half hour and filter. This mordant will be ready for use as soon as prepared and will keep for 10 days or longer. After a few days a second filtering may be necessary. It may be used without adding the hydrogen peroxide,but must be allowed to stand 2 or more days for ripening. When fresh it is a deep, purplish wine color, but on oxidation in the air or on adding hydrogen peroxide it becomes a brownish black.

### Stain

| | |
|---|---|
| Basic fuchsin, saturated alcoholic solution | 10 cc. |
| Anilin oil (1 part) and 95% alcohol (3 parts) mixed | 5 cc. |
| Water, distilled | 30 cc. |
| Acetic acid, 4 % | 1 cc. |

This stain can be made from stock solutions and usually works best if not more than 3 or 4 days old. It has given better results than carbol fuchsin. Gentian violet, and possibly other dyes, may be substituted for the basic fuchsin, but frequently is not as satisfactory as the fuchsin.

### Cultures

Cultures 20 to 24 hours old are generally the best. Transfers should be made daily to bouillon until the bacteria are found to be actively motile, then to agar (1½%) slopes. Or transfers may be made alternately from bouillon to agar, but the last transfer should always be to agar. Finally the bacteria should be transferred to sterile water and allowed to diffuse for 20 to 30 minutes. It seems to make a difference sometimes whether one uses tap water, distilled water, or slightly acidulated water. Loopfuls of the suspension may now be transferred to clean, flamed slides. If the droplets are allowed to evaporate *without spreading* most of the bacteria will be found where the edges of the droplets were, and frequently they will be freer from films there than elsewhere.

For class work mixed cultures of bacteria may be obtained by covering seeds such as beans with tap water and allowing the preparations to stand 24 hours or longer. If a thin scum forms on top it should be removed. Then smears may be made on slides, using one loopful for each, and stained. Some forms showing flagella are almost certain to be present in such preparations. Large spirals may sometimes be found in stagnant water.

### Staining

Treat with the mordant 2 to 4 minutes and wash with 6 or 8 changes of water poured on the slide. Then stain 2 to 3 minutes, wash thoroly, dry and examine. A cover glass is unnecessary. In staining the reaction may be varied by adding acid or alkali to the films and then dropping on the stain. I have found a solution of acetic acid (1:250) or of sodium hydroxide (1:750) satisfactory. One to four drops of the acid or 1 to 2 drops of alkali may be added to 6 drops of stain. An acid reaction is often the best and sometimes it is well to acidulate slightly the water used for washing.

The method described has proved very satisfactory for staining flagella of such forms as *Bacillus carotovorus*, *B. megatherium*, *B. radicicola*, *B. subtilis*, *B. vulgatus*, *Bacterium tumefaciens*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Pseudomonas pyocyanea*, and mixed cultures of large spirals and rods. Altho smears have been made repeatedly from cultures growing in liquids such as diluted bouillon, malt extract, autolized yeast, synthetic media, etc., I have rarely found stained flagella in such preparations, excepting the mixed cultures (with seeds) mentioned above.

### Staining the Yeast Nucleus

Various methods of staining the yeast nucleus may be found in the literature. I find that by the following rapid process the nucleus may be demonstrated in some kinds of yeast, *Saccharomyces ellipsoides* being an excellent species for this purpose. Cultures 3 to 6 days old should be used, smears made on slides and as soon as the water has evaporated fixed by passing three times thru a flame. Then stain in a rather concentrated aqueous solution of acid fuchsin (1 minute), wash off with 5% tannic acid (20 seconds), wash and destain with water slightly acidulated with acetic acid. Dry and examine with an immersion objective.

A mixture of equal parts of acid fuchsin and methyl green in water is also very satisfactory for staining the yeast nucleus. Basic dyes, such as safranin, basic fuchsin, gentian violet, methyl blue and brilliant green are not satisfactory. Some other acid dyes (eosin, rose bengal) stain the nucleus fairly well, but no other that I have tried as well as acid fuchsin.

### Staining Vegetative Cells and Spores of Bacteria and Yeasts.

According to Gutstein's studies on the ectoplasm of bacteria (1924–26) if one treats a smear of Gram-positive bacteria with a solution of tannin and then stains it with almost any basic dye, the ectoplasm alone will take the stain. Acid dyes, even after treatment with tannin, in general do not stain the ectoplasm. If Gram-negative

bacteria are treated with tannin followed by a basic dye they will stain either very slightly or very deeply, depending on the species, the time and the dye. If Gram-positive bacteria are stained with a basic dye, treated with a solution of tannin and counter-stained with a second basic dye of contrasting color, the body of the cell will be stained with the first dye and the ectoplast with the second one. But if Gram-negative bacteria are thus treated they will be stained either lightly or deeply with the second dye only. Gutstein also gives methods of staining capsules and spores of bacteria, and vegetative cells and spores of yeasts. I have used these methods for some time for differential staining of vegetative cells and spores of Gram-positive bacteria and of yeasts, and have obtained excellent results. Gutstein's general procedure is as follows: Smears are fixed by means of heat (3 times) and stained 2 or 3 minutes with a basic dye; they are then washed with water, treated for 2 minutes with a solution of tannin, washed with water, counter-stained, washed and examined. A 5% aqueous solution of tannin and 1% aqueous solution of basic dyes are used. Such dyes as methylene blue, brilliant green, safranin, basic fuchsin, gentian violet and methyl violet are recommended. Some of Gutstein's procedures that I have followed considerably and found satisfactory may be indicated briefly. For vegetative cells of bacteria (1) tannin followed by safranin, especially for forms that stain readily, such as *Sarcina lutea;* (2) carbol methylene blue, tannin, safranin; (3) carbol fuchsin, tannin, methylene blue; (4) carbol fuchsin, tannin, brilliant green. For bacterial spores (1) carbol fuchsin, 5% acetic acid, tannin, methylene blue; (2) same as (1) excepting brilliant green for methylene blue; (3) carbol methylene blue, acetic acid, tannin, basic fuchsin. For bacterial capsules (1) carbol methylene blue, tannin, basic fuchsin or safranin; (2) carbol fuchsin, acetic acid, tannin, methylene blue. For vegetative cells of yeasts (1) tannin, safranin; (2) carbol methylene blue, tannin, safranin; (3) methylene blue, tannin, safranin. For spores of yeasts (1) carbol fuchsin, acetic acid, tannin, methylene blue; (2) carbol methylene blue, acetic acid, tannin, safranin. Ziehl-Nielson's carbol fuchsin is apt to stain very deeply, so I prefer, even in staining vegetative cells, to wash quickly (5–10 sec.) after this stain with 95% alcohol. Carbol methylene blue or other carbol stains may be prepared by adding 5% of carbolic acid to a 1% aqueous solution of the dye. Addition of a little carbolic acid to the solution of tannin will prevent growth of molds and will not interfere with its usefulness. Gutstein also recommends treatment with 30% tannin for staining Gram-negative organisms. I have never obtained satisfactory results (differential staining) with this method.

When staining spores of either bacteria or yeasts I usually obtain better results by substituting 2 or 3% sulfuric acid for 5% acetic acid. It is often difficult to have spores of bacteria in good condition for class use. If the spores are too old they may stain only faintly by the usual procedure. I have found that if spores are first boiled 10 minutes in water, and then stained, uniformly well-stained smears result, even if the cultures are several days old or in some cases as much as 2 or 3 weeks old. By means of a needle one may transfer a growth from an agar slope to a test tube containing 2 to 4 cc. of sterile water, and then keep the test tube in a bath of boiling water for 10 minutes. Smears may be made, heated as usual (12 times thru a flame) and carbol fuchsin added *immediately*. The hot slide will heat the stain sufficiently. After staining 3 minutes, wash with water, treat with sulfuric acid 3 to 5 seconds, wash, treat with tannic acid 2 minutes, wash, stain 1 to 2 minutes with methylene blue and wash. The spores will be stained bright red and the old cell wall decidedly blue.

In an earlier note (1924) the writer stated that in staining spores of yeast with carbol fuchsin, sulfuric acid and methylene blue, the parts surrounding the spores stained only slightly. If, however, one treats the preparation with tannic acid, as outlined above, the ascus will stain quite deeply. I have never found it necessary to boil yeast spores in order to stain them. It is also unnecessary to use more heat than there is in the slide after fixation.

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## NOTES ON TECHNIC

NOTE ON LOEFFLER'S METHYLENE BLUE.—Reports have frequently been received during the last few years concerning the failure of methylene blue solutions to keep. Apparently the experience has usually been obtained when employing the well-known Loeffler formula, and the trouble has been too great polychroming of the methylene blue.

The Loeffler formula calls for a weak solution of potassium hydrate as a solvent. It was proposed back in the early days of bacteriology when the methylene blues available were either in whole or in part the zinc chloride double salt. The presence of excess zinc chloride would have caused a tendency for such methylene blues to be more acid than the free hydrochloride which is sold for staining purposes in this country today. As basic dyes do not stain well in acid solution, the presence of weak alkali, as in the Loeffler formula, was an undoubted advantage. As the modern methylene blues do not require neutralization, the Loeffler formula is no longer called for.

It is, furthermore, well known that the presence of alkali hastens the polychroming of methylene blue, that is its oxidation into the lower homologs, the azures. To a certain extent their presence improves the staining qualities of methylene blue; but as most American methylene blues at present contain a sufficient quantity of these lower homologs, further polychroming is ordinarily undesirable. This fact undoubtedly explains why the Loeffler formula, as made up today, has poor keeping qualities.

It is strongly recommended, therefore, that in using any methylene blue now on the market certified by the Commission, instead of the Loeffler formula, one employ an alcoholic solution of methylene blue diluted in distilled water instead of dilute alkali. A satisfactory formula is the following:

| | |
|---|---|
| Methylene blue, medicinal, (90% dye content) | 0.3 g. |
| Ethyl alcohol, 95%. | 30. cc. |

Dissolve and dilute in 100 cc. of distilled water.

—H. J. CONN, Geneva, N. Y.

DIFFERENTIATION OF SAFRANIN IN CYTOLOGICAL WORK.—A proper agent for the differentiation of safranin in cytological work has sometimes been a problem. Various combinations of hydrochloric acid with alcohol or water have been recommended, but the writer has found that some acid dye, such as light green SF yellowish, is preferable to hydrochloric acid. In the case of light green a 0.2%

solution of the dye in 95% alcohol is employed. If the green color is undesirable it is easily removed by a quick washing in water; or if the preparation is to be stained with Flemming's triple stain, the subsequent treatment with gentian violet removes the green. Using this method of decolorizing, cytoplasm appears unstained, while the chromatin in the division figures is colored bright red.

—RACHEL HAYNES, Geneva, N. Y.

## LABORATORY HINTS FROM THE LITERATURE

Under this heading will be given in each issue of the journal interesting new methods in microscopy that have been noticed in other publications. The readers' coöperation is urged in calling the editor's attention to any outstanding methods in this field that are overlooked.[1]

BAGINSKI, S. Sur la detection histochimique de l'adrenaline. Bull. d'Histol. et de Tech. Micr., **5**, 129. 1928.

Former methods for the demonstration of adrenaline in the tissues did not fix the tissues but tended to break them down. The following formulae act as fixatives for the tissue as well as reagents for the detection of adrenaline.

Formula 1:

2% ammonium chromate—30cc.
1.25% silver nitrate—20cc.
ammonia (0.912 sp. gr.)—3–4 drops

Formula 2:

2% ammonium bichromate—30cc.
1.25% silver nitrate—20cc.
ammonia (0.912 sp. gr.)—7–8 drops

These solutions keep well. Fix for 3–24 hours according to the size, but pieces should not be larger than 2–3 mm. Wash in distilled water until there is no more trace of chromium. Dehydrate slowly, clear in chloroform or xylol, not in cedar oil. Sections may be stained with various dyes. When natural adrenaline is diluted one to ten or fifteen million and is treated with these reagents, a clear test for adrenaline is obtained in the form of a fine black precipitate.

HARLOW, W. M. A chlorination method for macerating woody tissues. Bot. Gaz., **85**, 226–227. 1928.

The following method preserves the details of cell structure well: (1.) "Split the material match stick size. (2) Boil to expel air. (3) Immerse in strong chlorine water for two hours. (4) Wash. (5) Immerse in hot 3% solution of sodium sulfite for 15 minutes. (6) Wash. (7) Repeat the process from number three until, upon mild shaking, sufficient material separates for the purpose in view. When the red color first obtained upon the addition of the sodium sulfite fails to reappear in this reagent after several chlorinations, the wood should be completely delignified."

JOYET-LAVERGNE. Sur quelques procédés de recherche microscopique du glutathion dans les cellules. Comp. Rend. Soc. Biol., **93**, 658–660. 1928.

Glutathion is recognized by a light violet to red color produced in presence of sodium nitroprussiate in alkaline solution. *Direct method for fresh tissues:* Place fresh tissue on slide in drop of fresh 5% aqueous solution of sodium nitroprussiate, add drop of concentrated ammonia (N/1 NaOH for plant tissues), then put on cover glass and examine. This method shows glutathion in the reduced form but does not always give its exact location in the cell. Results may be confirmed with ferric chloride and picric acid, which show the reducing power of the SH-group, ferric chloride after reduction giving a black or gray color and picric acid a rose color. *Indirect method* (*using an intensifier*):1) Tissues are treated first in 10% aqueous potassium cyanide 5 minutes, then on slide with 5% aqueous sodium nitroprussiate. Glutathion regions are colored red. 2) (For plant tissue). After 10 minutes in saturated aqueous solution of sodium sulfite, treat on a slide with drop of sodium nitroprussiate and one of concentrated ammonia. Tissue may also be treated: 3) with saturated solution of ammonium sulfate for 15 minutes, or 4) with 2% trichloracetic acid for 5 minutes and then as above under (2). For fixed tissue employ one of following fixatives: 1) 2% trichloracetic acid; 2) saline formol (formalin 15 pts., physiological salt solution 75 pts.);

[1]Thru coöperation with The Wistar Institute of Anatomy and Biology, Philadelphia, Pa., it has been arranged for these same methods to appear on the Laboratory Service Cards of The Wistar Institute bibliographic Service. Those desiring these procedures printed on cards but not desiring all cards of The Wistar Institute Bibliographic Service may obtain them by sending their order direct to Stain Technology. Cards will be mailed post paid for 6 cents each.

3) absolute alcohol. Fix 24 hours. After the first two fixatives treat tissues by (1) or (3) of the above methods. After absolute alcohol place in 10% potassium cyanide in 70% alcohol for 10 to 15 minutes. Put on slide and add drop of 5% sodium nitroprussiate in 50% alcohol.

LILLIE, R. D. Kalkfärbung mit nachfolgender Entkalkung, eine neue Methode. Zeit. f. wiss. Mikr., **45**, 380–381. 1928.

Fix in 10% formol, wash in distilled water frequently changed. Immerse in 2–2.5% silver nitrate in the 37° incubator for 4 or 5 days. Wash well with distilled water. For the subsequent decalcification use formic acid, hydrochloric acid with formol, hydrochloric acid alcohol, or von Ebner's hydrochloric sodium-chloride mixture. The latter is the best. Deacidify in half saturated sodium chloride solution, renewing it frequently. Wash in running water. Dehydrating, clearing and embedding follow as usual. Bones in the small lamellae will be colored wholly black, in the thicker ones, the outer layers will be dark, the deeper ones will have dark bone cells and Sharpey fibers. The calcified cartilage and other calcified tissue will also be darkened. Dental enamel is only partially dyed, dentine on the other hand is fully darkened. The calcium phosphate is changed to silver phosphate by the action of the silver nitrate. Since this is dissolved by nitric acid and sulfuric acid, one must decalcify with hydrochloric or some other acid that does not dissolve silver phosphate. Hydrochloric acid converts the silver phosphate into silver chloride which is almost insoluble.

MASCRE, M. Sur la fixation de la chondriome de la cellule végétale. Comp. Rend. Acad. Sci., **185**. 866–869. 1927.

For chondriome fixation neutralized formol or chromic osmic mixtures are good, but acetic is not. Nitric acid works, probably because it is a good protein coagulant. Since trichloracetic, monochlor acetic and cyanacetic acids also coagulate protein, they were studied as fixatives for the chondriome. Material: shoots of *Elodea canadensis*, barley and bean, *Hemerocalis* flowers, stamens of *Arum maculatum*. Fixatives: 5% acetic, 3% monochlor acetic, 3% cyanacetic, 2% trichloracetic, both in water and in 20% neutralized formol. Material fixed two to six days was either washed quickly in water to avoid swelling or was treated 15 days with 3% potassium bichromate. All material was dehydrated, cleared and embedded in paraffin. Stains: Altmann's acid fuchsin with picric acid, Heidenhain's or Regaud's iron hematoxalin.

The acid solutions without formalin fix plastids but destroy chondriocontes. With acetic and monochloracetic acid the chondriocontes are transformed into large vacuoles: with cyanacetic, trichloracetic, and formol acetic (pH 1.9) into smaller vacuoles. Twenty percent formol plus 3% monochlor acetic (pH 1.6), cyanacetic (pH 1.3) and 2% trichloracetic (pH 0.6) give the best chondriome fixation, equaling Regaud's method. Treatment with potassium bichromate is unnecessary after these fixatives, and staining is shortened to six hours in 3% iron alum and twelve hours in hematoxylin.

MILLOT, J. and GIBERTON, A. Sur utilization, en histologie, des pièces conservées dans le formol, pour la mise en évidence des graisses, Comp. Rend. Soc. Biol., **97**, 1674, 1927.

Pieces of tissue fixed and kept for some time in formaldehyde solution do not stay in good condition for histological study, as has been popularly supposed. Histologic examination of pieces of fatty tissues fixed in this reagent and withdrawn every three weeks shows a progressive diminution of the fat compounds, neutral fats, and lipoids, even to their complete disappearance, whatever the method used to demonstrate them. On the other hand there is an augmentation of free fatty acids as determined by Fischler's copper acetate method. Various tissues were used (liver of fish and of mammals, kidney of cat and dog) and the results vary only in the speed with which they occur. For example, with fresh pieces of liver of Syngnathes in fresh cells quite a large amount of fat may be seen. After fixation 24 hours in formol (20 pts. commercial solution to 80 pts. water) and keeping for three months in a weak formol solution (5 to 95) cells do not contain a trace of fat. The same results are observed when the tissues are preserved in stronger solutions of formalin (25–100).

The explanation for the disappearance of the fat seems to be that the lipase present in the tissue is capable of acting in spite of the presence of the formaldehyde. Two things support this point of view; 1) disappearance of fat in mammals is more rapid

when tissues are preserved at temperature from 35° to 40°; 2) destruction of fats takes place faster in some organs than in others and it is fastest in organs where fat is ordinarily broken down most rapidly.

CRAIGIE, J. A method of staining bacterial flagella. Brit. J. Exper. Path., 9, 55–7. 1928.

The writer proposes the following modification of Zettnow's silver impregnation method: Suspensions of 24-hour cultures are fixed in formol (physiological saline, or buffer solution at pH 7, containing 2% formalin). Leave in fixative 1 hour or over night. Glassware is cleaned in bichromate-sulfuric-acid. Dilute formolized suspensions in distilled water and spread a small loopful over a cleaned slide. Dry at 37°C. Heat at 90–100° for 5 minutes or longer. Place in distilled water 5 minutes, then wash in distilled water. Dry and heat again at 90–100°. *Mordanting:* Use Zettnow's mordant (10 g. tannic acid, 200 cc. distilled water, heated to about 60° and 30 cc. of 5% aqueous tartar emetic added slowly). Flood slide with mordant and heat 5–10 minutes at 90–100°; watch constantly to prevent drying at edges by adding more mordant as required. Wash under tap, then in distilled water. *Silvering:* Dissolve 1 g. silver sulfate in 250 cc. distilled water, and to 20 cc. of the solution add mono-ethylamine solution (33% B. D. H.) until opacity just clears up. Flood slide with this and warm till steaming begins, holding at mild heat till preparation turns brown or black—but no longer. Wash under tap, then in distilled water. *Gold toning:* Add 20 drops of 1% gold chloride solution to 20 cc. distilled water. Immerse slides in this for 30 minutes or longer. Wash and dry.

GAUSSEN, C. Coloration panotique d'exécution rapide permettant d'opérer sur la même préparation l'examen cytologique l'examen bactériologique, et la recherche du Bacille de Koch. Comp. Rend. Soc. Biol., 97, 1657–8. 1927.

This method stains Koch's bacillus, other organisms, and cells in sputum and is usefu for pathologic serous exudates, cephalic-spinal fluid and urine. Make thin film on slide dry at 37°, fix in ether-alcohol, place on hot plate and stain five minutes in Ziehl's solution, made up by triturating in a mortar 1 g. basic fuchsin, 5 g. phenol, and 10 cc. 95% alcohol; add 90 cc distilled water and filter. After staining wash in running water, dry, stain for 4 minutes in a differentiating stain made up as follows. Dissolve separately (warm) in round bottom flasks:

(1). 2.5 g. Methylene blue, 150 cc. 80% alcohol, 3 g. lactic acid;

(2). 2 g. Orange G, 100 cc. 80% alcohol, 2 g. lactic acid.

Put aside 50 cc. of the blue solution to cool. Mix the rest of blue with orange, heat again, cool, filter. Heat precipitate remaining on filter with 30 cc. 80% alcohol. Cool, filter, mix two filtrates and add the 50cc. of blue solution that has been put aside, filter. (Writer gives no information as to the source of these stains.)

Nuclei are stained violet, acidophil protoplasm yellow, basophilic blue, eosinophil granulations greenish, mucus violet, sero-albuminous exudates yellow, microörganisms blue-green against yellow cytoplasm; Spirillae are gray, Diplococci deep violet red, common acid resisting organisms deep maroon, Koch bacillus bright red.

HEWER, E. R. The activity of the thyroid gland in relation to the staining reactions of the colloids. J. Path. and Bact., 30, 621–5. 1927.

The degree of activity of the stored thyroid secretion may be estimated by the staining reaction of the colloid. Freshly secreted active material gives an alkaline staining reaction and old or inactive material an acid one, using hematoxylin and biebrich scarlet, Weigerts resorcin-fuchsin, anilin-blue-orange G, hematoxylin and congo red, or hematoxylin and benzopurpurin. These results check with the degree of activity of the gland estimated by the pathologist. No information is given as to the sources of the dyes employed in this work. Determination of the iodine content of the thyroids shows that the variation in staining does not depend on the iodine content but on the acid-alkaline reaction of the gland.

KATER, J. M. Cytology of *Saccharomyces cerviciae*. Biol. Bul., 52: 438–448. 1927.

The following technic is described: Pure cultures of *Saccharomyces cerviciae* are cultivated on French proof broth and French proof agar. Organisms are transferred to slides previously smeared with albumin fixative and moist films were fixed with corrosive-acetic-alcohol or preferably in Bouin's fluid. Stains used were iron alum

hematoxylin with or without light green as a counterstain. Delafields' hematoxylin, carbol fuchsin, eosin, and orange G did not give good results. No information is given as to the source of any of the dyes employed. Best results were obtained with Bouin fixation and staining with iron alum hematoxylin before washing out all of the picric acid. This technic prevents the cytoplasmic chromatic material from staining as heavily and thus the nucleus shows up well. Light green is useful in staining the metachromatic grânule, and also the gelatinous substance sometimes secreted by the yeast cells. Fixation and staining in carbol fuchsin followed by light green showed the alveolar structure of the cytoplasm.

KELLEY, A. P. and SHOEMAKER, M.B. Staining of yeast cells. Bot. Gaz., **83**, 318, 1927.

This is a new method for class use which is simple but gives good results. Place a drop of Mayer's albumin fixative diluted one to ten with distilled water on a clean slip. Stir in a small drop of the yeast culture and allow to dry over gentle heat. Stain for fifty seconds in acid fuchsin, wash, dry, and mount in balsam.

LA CORTE, J. G. Influence du pH dans les colorations par la méthode de Giemsa. C. Rend. Soc. de Biol., **98**, 1579–80, 1928.

Distilled water for diluting Giemsa staining solution was buffered with enough sodium acid phosphate to obtain a pH-range from 6.6–7.8. For staining one drop of Giemsa solution was added to 1 cc. of the buffer solutions. Blood smears of guinea pig experimentally infected with *Trypanosome gambiense* were stained with these solutions from ½ hour to 24 hours.

Optimum staining was obtained with solutions at pH 7.4 for one hour. With solutions at pH 6.6, 6.8, and 7.2 a reddish staining was obtained and differentiation was poor. With solutions at pH 7.6 and 7.8 the color was too blue.

SOUTHGATE, H. W. Note on preparing muci-carmine. J. Path. and Bact., **30**, 729 1927.

Precise directions for preparing Mayer's muci-carmin which give constant results are given. One gram of carmin, 1 g. of dry powdered aluminum hydroxide 100 cc of 50% alcohol, 0.5 g. of powdered anhydrous aluminum chloride are placed in a 500 cc. flask which is placed on a boiling water bath with frequent shaking. The mixture is boiled exactly 2½ minutes, cooled under the tap and filtered. For staining dilute 1 cc. of this solution with 9 cc. of distilled water and stain sections 15–20 minutes. Rinse with distilled water then treat with alcohol, xylol and balsam. This stain colors mucin deeply. The writer has thus prepared solutions from samples of carmin obtained from the British Drug Houses, from G. T. Gurr, and from Grübler (pre-war).

WEISS, E. Method of staining flagella. J. Inf. Dis., **43**, 228–231. 1928.

A method of staining bacterial flagella is proposed, as follows: To an 18-hour broth culture glacial acetic acid is added (q. s. ad 5%). Keep tube in water bath at 37°C for 15 minutes. Transfer a loopful to a slide and dry. Mordant for 2–5 minutes, hot, in a tannic-acetic-acid mordant:—100 g. tannic acid in 100 cc. 95% alcohol (use one part) plus 7.5 cc. glacial acetic acid in 100 cc. commercial formalin (use two parts). Wash with warm water 1 to 2 minutes. Stain with a basic dye (preferably 5% gentian or crystal violet in formalin). If the concentration of the basic stain is not higher than 1% stain cold 4 to 6 minutes or steaming for 2 to 3 minutes; if higher than 1% stain only 1 to 2 minutes. Wash with water and cover with a solution of an acid dye for 10 minutes (preferably acid fuchsin, acid green, or acid violet; it is not specified which of the various acid greens or what acid violet is employed). Wash and dry. The author tries various acid and basic dyes, but does not state the source from which they were obtained.